

## Development of Host- and Symbiont-Specific Monoclonal Antibodies and Confirmation of the Origin of the Symbiosome Membrane in a Cnidarian–Dinoflagellate Symbiosis

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**Abstract.** The “symbiosome membrane” as defined by Roth *et al.* (1988) is a single, host-derived membrane that surrounds an endosymbiotic organism, separating it from the cytoplasm of the host cell. However, in the case of cnidarian–dinoflagellate endosymbioses, clear identification of the symbiosome membrane is complicated by the fact that each algal symbiont is surrounded by multiple layers of apparent membrane. The origin and molecular nature of these membranes has been the subject of considerable debate in the literature. Here we report the development of host-specific (G12) and symbiont-specific (PC3) monoclonal antibodies that allow separation of the host and symbiont components of these multiple membranes. Using immunocytochemistry at both the light and the electron microscopic level, we present data supporting the conclusion that the definitive symbiosome membrane is a single, host-derived membrane, whereas the remainder of the underlying apparent membranes surrounding the algal cell are symbiont-derived. The potential for macromolecules associated with these membranes to act as cellular signals critical to recruiting symbionts and maintaining established symbioses is discussed.

### Introduction

Endocytobiotic associations (symbioses in which one organism lives inside the cell cytoplasm of another organism) are fairly common in nature. They include many prokaryote–eukaryote associations (Jeon and Jeon, 1976;

Lerouge *et al.*, 1990; Regensburg-Tuink and Hooykaas, 1993; Udvardi and Day, 1997; Ferrari *et al.*, 1999) as well as a wide variety of eukaryote–eukaryote associations including parasite–vertebrate (Sibley *et al.*, 1986; Hall *et al.*, 1991; Russell *et al.*, 1992; Sam-Yellowe, 1992; Collins *et al.*, 1997; Forero *et al.*, 1999) and microalgal–invertebrate (for reviews see Lee and Anderson, 1991; Trench, 1993). A common feature of many of these associations is the membranes that surround the microsymbiont, effectively separating it from the cytoplasm of the host cell. These membranes form the boundary across which all cell-to-cell communication between the symbiotic partners must occur; thus the membranes become the critical interface for communication and control between symbiont and host (Day and Udvardi, 1992). An understanding of how these membranes mediate interactions between the partners of a symbiotic association requires a knowledge of their origin and molecular nature.

In an attempt to consolidate endosymbiotic research so that homologies in all the systems could be investigated, Roth *et al.* (1988) introduced the terms “symbiosome” and “symbiosome membrane” to describe the intracellular symbiotic compartment in which symbionts reside. In zooxanthellae–cnidarian symbioses, the symbiosome is commonly recognized to include a continuous outer, or vacuole, membrane underlain by multiple layers of additional membrane that surround the “pellicle,” or cell wall, of the internal symbiont (Battey, 1992). The origin and nature of these membranes have been debated since they were first described. Taylor (1968) stated that some of these membranes were formed from host tissue, but others were contributed by the symbiont itself; however, he also stated that deter-

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mining which membranes came from which source was difficult. Dodge and Crawford (1970), in their investigation of the "periplast" of zooxanthellae, agreed with Taylor, stating that "it is impossible to tell whether these [membranes] belong to the host or alga." Undaunted by this proclamation, many papers have been published suggesting possible origins for these membranes. These suggestions have naturally centered on two possible origins: (1) the host cell (Colley and Trench, 1983; Palincsar *et al.*, 1988; Rands *et al.*, 1993; Tripodi and Santisi, 1982), and (2) the algal cell (Kevin *et al.*, 1969; Taylor, 1971; Trench and Winsor, 1987; Wakefield *et al.*, 1998, 2000). Unfortunately, in all cases, suggested origins are presented without any supporting experimental evidence.

In this study, immunocytochemical methods were employed to obtain evidence needed to support or dispute the claims of symbiont *versus* host origins of the multiple layers of apparent membrane surrounding the endosymbiont. The marine anthozoan *Aiptasia pallida* and its endosymbiont *Symbiodinium bermudense* (Banaszak *et al.*, 1993) were used in confocal and electron microscopic investigations that employed two monoclonal antibodies produced against antigens associated with the membranes surrounding the algal symbiont. The data obtained provide the experimental evidence needed to reject one of the proposed hypotheses for the origin of the multiple layers of membrane. The significance of these findings is discussed.

## Materials and Methods

### *Animal collection and care*

Specimens of *Aiptasia pallida* were collected in the Florida Keys and transported to Auburn University, Alabama. Collected animals ranged in length from about 4.0 to 8.0 cm, with an oral disc radius of about 2.5 to 5.0 cm. The animals were maintained in two sets of established 300-gallon aquarium systems with both external trickle and undergravel filtration. Artificial seawater for the aquaria was prepared with deionized water and Reef Crystals (Aquarium Systems) at a salinity of about 30 ppt. One aquarium system was maintained on a 12-h light/12-h dark cycle; the other was kept in total darkness. The constant dark condition causes a gradual loss of all photosynthetic symbionts within the host cells; these bleached animals are said to be aposymbiotic. Animals were fed with newly hatched brine shrimp nauplii every other day. Tank temperature ranged between 25 and 30 °C. Individual animals reproduce asexually by producing pedal lacerations: small pieces of tissue that pull away from the pedal disc and grow into a new individual. Thus, within the aquaria, animal sizes ranged from less than 0.5 to about 8 cm in length.

### *Monoclonal antibody production*

**Antigen preparation.** Eight specimens of *Aiptasia pallida*, each about 5.0–8.0 cm long, were removed from the aquaria. Two specimens were placed in 6.0 ml of a homogenization solution consisting of 1.0 mM phenylmethylsulfonyl fluoride (PMSF) in 0.45- $\mu$ m Millipore-filtered seasoned aquarium water (MFSAW) pre-cooled to 4 °C. The tissues of the specimens were fully dispersed using a Handishear (Virtis) tissue homogenizer; an ice bath was used to maintain the pre-cooled temperature. This process was repeated for all eight specimens, which were then combined and split into four samples. Each sample was poured into 15-ml glass centrifuge tubes and centrifuged at about 250  $\times$  g for 3 min to pellet the extracted symbiosomes. The supernatant was discarded, and the pelleted symbiosomes were resuspended in homogenization solution. This process was repeated three times until a firm pellet of extracted symbiosomes was obtained in each tube. Each symbiosome pellet was resuspended in 1.0 ml of nanopure filtered water (np-H<sub>2</sub>O) and either remained untreated or was processed in one of the following ways: (1) fixed in 4% paraformaldehyde containing 0.2 M Millonig's phosphate buffer (MPB) for 10 min and then rinsed three times in 0.2 M MPB and finally resuspended in np-H<sub>2</sub>O, (2) boiled for 1 min, or (3) repeatedly frozen and thawed by placing the sample in an Eppendorf tube, dropping it into liquid nitrogen, removing and warming until it thawed, and then repeating. All four types of samples were then combined to produce about 4.0 ml of "symbiosome slurry." This slurry was used as a source of antigen for the production of antibodies.

**Antibody production.** The production of monoclonal antibodies followed standard procedures (Kohler and Milstein, 1975). Briefly, BALB/C mice were injected subcutaneously with 0.1–0.2 ml of the symbiosome slurry in Freund's complete adjuvant. At 3 weeks and 6 weeks, symbiosome slurry in Freund's incomplete adjuvant was injected into the peritoneal cavity. Seven days after the last injection, blood was taken from the tail vein of the inoculated mice. Blood sera were titered 1:50 through 1:10,000 and placed in a 96-well plate coated with fixed symbiosomes to undergo an enzyme-linked immunosorbent assay (ELISA) for the presence of symbiosome antibodies.

Once levels of serum antibodies were sufficient, a mouse was selected and sacrificed. Lymphocytes were removed from the spleen, washed to remove red blood cells, and then suspended in RPMI 1640 tissue culture medium (RPMI). Throughout the fusion procedure, this suspension was maintained at 37 °C by using a dry heating block. A 4:1 mixture of lymphocytes and AG-8.653 myeloma cells was added to a sterile centrifuge tube and pelleted. The supernatant was removed, and a solution of 50% polyethylene glycol in 20 mM phosphate buffered saline (PEGM) was slowly added while the cell suspension was constantly stirred. Additional

RPMI was then added while stirring continued. After several minutes the suspension was again centrifuged to pellet the cells, and the PEGM + RPMI medium was removed and replaced with RPMI supplemented with BSA (15% bovine serum albumin), OPI (0.15% oxaloacetate, 0.05% pyruvate, 0.0082% bovine insulin), and HAT ( $5 \times 10^{-5}$  M hypoxanthine,  $2 \times 10^{-7}$  M aminopterin,  $8 \times 10^{-6}$  M thymidine). The resulting fusion mixture of cells was aliquoted in 100- $\mu$ l amounts into the wells of 96-well plates that were then incubated at 37 °C.

Developing cell cultures were periodically fed and checked as they increased in size. Once the 96-well plate cultures had reached one-third confluency they were screened by an immunofluorescent dot-spot test to identify culture wells that were producing antibodies against symbiosome antigens (see below).

**Screening.** Symbiosomes that had been extracted using the above-outlined procedure were fixed in 4% paraformaldehyde and 0.2 M MPB for 10 min. Fixed symbiosomes were rinsed three times in 0.2 M MPB and then resuspended and diluted in np-H<sub>2</sub>O. Spots of fixed symbiosomes were dried onto spot slides. Each spot was incubated first in 30  $\mu$ l of blocking media (BM) consisting of 5% heat-inactivated goat serum in 20 mM phosphate buffered saline solution containing 0.1% Triton X-100 (PBS+). Subsequently, spots were incubated in 30  $\mu$ l each of RPMI supernatant from cell-culture wells for about 1 h. Spots were then washed three times in PBS+ and incubated in goat anti-mouse fluorescence isothiocyanate (FITC)-conjugated secondary antibody (1:100 in BM) for 1 h, followed by three more rinses in PBS+. Spots were then examined with a Zeiss epifluorescence microscope. Cells in culture wells producing symbiosome antibodies were suspended in RPMI and cloned by limiting dilution. Upon reaching one-third confluency, clone wells were screened and positive wells cloned again. This process of recloning was repeated until all of the clones in a freshly inoculated 96-well plate showed the same degree of antigen recognition. The final outcome was the isolation of two hybridoma cell lines (G12 and PC3) producing antibodies that recognized antigens associated with the symbiosome membranes. The two cell lines were then expanded, allowing collection of antibody containing spent culture medium for immunohistochemistry and providing cells for frozen storage in liquid nitrogen.

The two cell lines were isotypized using an ISO-1 isotyping kit purchased from SIGMA chemical company. The G12 antibody was found to be an IgG, and the PC3 antibody was an IgM.

#### *Immunocytochemistry and confocal microscopy*

**Cultured symbionts.** Axenic cultures of *Symbiodinium microadriaticum* (Freudenthal, 1962) extracted from *Cassiopea xamachana*, *S. pulchrorum* (Banaszak *et al.*, 1993)

extracted from *Aiptasia pulchella*, and *S. bermudense* extracted from *A. tagetes* (= *A. pallida* Muller-Parker *et al.*, 1996) have been continuously maintained in incubation chambers within our laboratory for about 3 years (cultures were initially provided by Dr. William Fitt, University of Georgia). Incubation temperatures were maintained at 28.5 °C and illumination of the cultures was on a 12-h light/12-h dark cycle. Cultures were grown in 10 ml of ASP-8A culture medium (Blank, 1987) in sterile 15-ml culture tubes. New cultures were started from existing cultures roughly every 2 months, using sterile techniques.

Samples from each culture were removed and fixed in 4% paraformaldehyde in 0.2 M MPB. Fixed algal cell samples were rinsed three times in 0.2 M MPB and then incubated in the following solutions: BM for 1 h, PC3 primary antibody (1:100 in BM) or G12 primary antibody (1:100 in BM) for 3 h, rinsed 3 times in PBS+ for 10 min each, FITC-conjugated-IgM-specific secondary antibody (1:100 in BM) for PC3 antibody or FITC-conjugated-IgG-specific secondary antibody (1:1000 in BM) for G12 antibody for 1 h, followed by three rinses in PBS+ for 10 min each. Controls were also run using either BM only or a nonspecific antibody-producing hybridoma cell supernatant in place of the primary antibody solution.

**Extracted symbiosomes.** Symbiotic *Aiptasia pallida* specimens were homogenized, and the symbiosomes were extracted and fixed using the previously described procedures. Labeling of the fixed symbiosomes with the same controls was done using the procedures described above for cultured symbionts.

**Cryostat samples.** Small (0.5–1.0 cm) symbiotic and aposymbiotic specimens of *Aiptasia pallida* were fixed and rinsed following the same procedure as used on the extracted symbiosomes. After rinsing, these specimens were placed in a 1:1 mixture of 0.2 M MPB and Fisherbrand tissue freezing medium (TFM) and left overnight. Specimens were then removed from the 1:1 mixture and placed in 100% TFM. Specimens were frozen, and thin sections were cut using a Reichert-Jung 2800 Frigocut N cryostat. The sections were collected and placed on warm, 4% gelatin-coated slides. The tissues were labeled using the same procedures as those used to label extracted symbiosomes. Sections of symbiotic tissue and aposymbiotic tissue were tested for positive labeling using both PC3 and G12 primary antibodies. The same controls as those used on the extracted symbiosomes were used on the cryosectioned tissue.

**Whole-tissue samples.** Tentacles (2.0–3.0 cm long) from *A. pallida* were removed from symbiotic specimens. Small (0.5–1.0 cm) whole, aposymbiotic *A. pallida* specimens were also collected. Both types of tissue were fixed and rinsed following the same procedure as that used for extracted symbiosomes and sectioned tissue. After the final rinse, the aposymbiotic specimens were cut into small pieces (2.0–3.0 mm). Symbiotic tentacles were also cut into

small pieces (2.0–3.0 mm), and additional cuts were made longitudinally through the tentacle so that the symbiotic endodermal layer was fully exposed to the incubating solutions. Single- and double-labeling experiments using G12 and PC3 primary antibody were performed using the same procedures and controls as described above for extracted symbiosomes and sections.

**Imaging.** Labeled symbiosomes, sections, and whole-tissue samples were placed on glass slides and infiltrated with either glycerol mounting medium containing 4% *n*-propyl gallate (pH 9.5, Giloh and Sadat, 1982) or Permafluor aqueous mounting medium (Beckman Coulter). Fluorescent images were captured using a BioRad MRC-1000 laser scanning confocal microscope equipped with a krypton-argon laser. For those tissues that were labeled only with FITC-conjugated antibodies, the FITC-labeled tissue was visualized first with 488-nm laser excitation and a 500-nm fluorescent emission filter. A second image was then acquired of the autofluorescence of the algal chloroplast by using 514-nm laser excitation and a 660-nm fluorescent emission filter. When the filter sets provided with the confocal microscope are used, autofluorescence due to chlorophyll is very dim; thus it was necessary to maximize the gain to capture images of chloroplast fluorescence. Each image was saved, pseudocolored (green for FITC; red for autofluorescence), and merged using BioRad's confocal assistant. For double-labeled tissues the same procedures were employed as for single-labeled tissues, except that maximizing the gain for the FITC-labeled tissue was unnecessary because its fluorescence was much brighter than the apparent autofluorescence of the chloroplast.

To ensure that the labeling could be compared accurately, the positively labeled tissue was imaged first. The laser intensity, iris diameter, gain, black level, and enhancement were all optimized for the FITC label. After switching the filter set, each parameter (*i.e.*, laser intensity, iris diameter, *etc.*) was again optimized to capture either the autofluorescent algal chloroplast or the FITC label. Once both sets of parameters were established (one for FITC and the other for either autofluorescence or FITC label), they were not changed during the image acquisition of other experimental tissues.

### Electron microscopy

**Standard fixation and embedding.** Small pieces (2–4 mm) of symbiotic tentacle were removed from a live anemone and fixed in 0.5% glutaraldehyde + 4% paraformaldehyde + 0.2 M sucrose in 20 mM phosphate buffered saline (PBS) solution at 37 °C for 4 h. Tissues were rinsed in 20 mM PBS + 0.2 M sucrose three times for 15 min each time and dehydrated in an ethanol series (30%, 50%, 70%, 85%, 90%, 95%, 100%) for 30 min in each, at room temperature, with constant rotation. Tissues were then incubated in a 1:1

mixture of 100% ethanol and LR White and left overnight with constant rotation. The next day, tissues were placed in 100% LR White, incubated for 6 h with constant rotation, and then placed in fresh LR White in an aluminum pan; the pan was placed inside an acrylic plastic chamber and sealed. Intake and outflow valves were used to flood the chamber with N<sub>2</sub> and exclude oxygen. Finally, the sealed chamber was incubated at 70 °C overnight to polymerize the plastic.

**Cryofixation and embedding.** As above, small pieces (2–4 mm) of symbiotic tentacle were removed from a live anemone. Two or three pieces were placed in a brass planchet, inserted into a Balzers 010 HPM high pressure freezing machine, and quick-frozen. Frozen tissues were transferred to liquid N<sub>2</sub> and held there briefly before being transferred into a Leica EM AFS automatic freeze-substitution system. The tissues were placed in a freeze-substitution medium of methanol (MeOH) + 0.5% uranyl acetate. The freeze-substitution medium and tissues were maintained at –90 °C for 2 days before the temperature of the system was slowly ramped up 5 °C/h until it was stabilized at –45 °C. The freeze-substitution medium was removed and the tissues were rinsed twice, for 30 min each time, in pure MeOH. The tissues were then incubated at –45 °C in the following: a 1:1 mixture of MeOH:Lowicryl HM20 resin for 2 h, a 1:2 mixture of MeOH:HM20 for 2 h, pure HM20 for 2 h, and finally pure HM20 overnight. Tissues were placed into molds filled with fresh HM20 and polymerized at –45 °C by ultraviolet light over a 3-day period.

**Sectioning, labeling, and staining.** Blocks of the standard-fixed and cryofixed tissues were trimmed, and ultrathin sections were cut using a Reichert-Jung Ultracut E ultramicrotome. Sections were collected on carbon-coated, Formvar-covered slot and 100-mesh grids. Grid-mounted sections were incubated on drops of the following solutions: 0.05 M glycine in incubation buffer (10 mM phosphate buffer + 150 mM NaCl + 0.1–0.2% Aurion BSA-c [acetylated bovine serum albumin] + 20 mM NaN<sub>3</sub>) for 15 min, blocking medium (incubation buffer + 5% BSA + 0.1% cold water fish skin gelatin + 5% heat inactivated goat serum) for 30 min, incubation buffer 3 × 5 min, diluted primary antibody (see below) in incubation buffer overnight, incubation buffer 6 × 5 min, immunogold-conjugated secondary antibodies (see below) diluted in incubation buffer for 2 h, incubation buffer 6 × 5 min, 10 mM PBS 3 × 5 min, postfixed in 2.5% glutaraldehyde in 10 mM PBS for 5 min and then rinsed in 10 mM PBS for 5 min, nanopure H<sub>2</sub>O 5 × 2 min, Aurion silver enhancement for 20 min, nanopure H<sub>2</sub>O (np-H<sub>2</sub>O) 3 × 5 min, saturated uranyl acetate in 50% MeOH for 20 min, and nanopure H<sub>2</sub>O 3 × 5 min. Grids were then carefully blotted by touching the edge of the grid to filter paper and allowing most of the water to be wicked away. Grids were then placed on filter paper and allowed to dry. Labeled tissues were viewed and photo-

graphed using a Zeiss EM-10 transmission electron microscope.

Primary antibody dilutions used in the procedure above were either PC3 1:100, G12 1:100, or both 1:100 in incubation buffer. Secondary antibodies used were goat anti-mouse (GAM) IgM-specific antibody conjugated to an ultrasmall ( $\sim 1.0$  nm) gold particle (Aurion), GAM IgG-specific antibody conjugated to an ultrasmall gold particle (Aurion), or a combination of the GAM IgM-specific antibody-ultrasmall gold with a GAM IgG-specific antibody conjugated to a 20-nm gold particle (ICN). Some sections were treated with protein A conjugated to a 15-nm gold particle (Aurion). Sections were incubated in both 1:10 or 1:100 dilutions of secondary antibody or protein A in incubation buffer.

Single-label controls included incubations in BM or non-specific primary antibodies in place of PC3 or G12. Controls for nonspecific cross reaction of the secondary antibodies were also done. This included incubation in PC3 followed by the IgG-specific secondary antibody and incubation in G12 followed by the IgM-specific secondary antibody. Double-label controls included incubations in BM, in nonspecific primary antibodies, in PC3 only, or in G12 only; each was followed by incubations in both secondary antibodies.

**Ultra-cryomicrotomy.** Small pieces (2–4 mm) of symbiotic *A. pallida* tentacle were removed from a live specimen. Tentacle pieces were fixed using the same protocol as that used in the standard fixation and embedding procedure described above. However, after the tissue had been rinsed it was placed in an 80% sucrose solution at 4 °C and left overnight. The following day the tentacle pieces were removed and placed on small metal stubs. Care was taken to remove excess sucrose solution, and then the stub and tissue were plunged into liquid nitrogen ( $\text{LN}_2$ ) until completely frozen. Following this initial freezing the tissues remained frozen at all times, either in  $\text{LN}_2$  or in the cold chamber of the ultra-cryomicrotomy apparatus.

The frozen tissue was trimmed and sectioned on the Reichert-Jung Ultracut E ultramicrotome fitted with an FC4E ultra-cryomicrotomy unit. Trimming was done at  $-70$  °C with glass knives; sections were cut at  $-100$  °C with a Diatome 45° angle ultra-cryomicrotomy diamond knife. A Diatome Static Line I was also used to reduce the build-up of static charge on the knife surface, allowing a fine ribbon of 75- to 85-nm sections to be produced. Each ribbon was pulled from the knife's edge with an eyelash brush and picked up by using a tiny drop of 80% sucrose suspended in a wire loop. (The sucrose drop begins to freeze as it is brought near the knife surface, and the tissue was picked up just before the drop was completely frozen. Thus the tissue was frozen onto the sucrose drop and removed from the knife.)

The drop was removed from the cryo-chamber and allowed to thaw. The drop was then applied, tissue side down,

to a 100-mesh, Formvar + carbon-coated nickel grid. The grid adhered to the drop so that it could be flipped over and floated off the wire loop onto a plate of 4 °C solidified 2% gelatin in np- $\text{H}_2\text{O}$ . Grids could be stored on the gelatin for several days before being used in the immunogold labeling procedure.

In preparation for labeling the grids, a small amount of the solidified gelatin was removed from around and under each grid. The grids and gelatin were placed on a small piece of Parafilm attached to a glass petri dish and placed in a 37 °C oven for about 30 min. When the plate was removed, the grids could be easily picked out of the now-liquid gelatin with fine forceps. The grids were immediately placed into the 0.05% glycine solution and were incubated in all of the same solutions used on the plastic-embedded tissues. After the final rinse in np- $\text{H}_2\text{O}$ , the grids were floated onto a solution of 2% methyl cellulose in np- $\text{H}_2\text{O}$  + 2% uranyl acetate (MU). The grids were then picked up with a wire loop that was just slightly larger than the grid itself, and the excess MU was wicked away by touching the edge of the loop to a piece of filter paper. Care was taken to ensure that only a thin layer of MU was left over the tissue. The MU was then allowed to dry completely, and the grids were removed from the loops and stored in grid boxes until they were viewed and photographed using the Zeiss EM-10 transmission electron microscope.

## Results

### Immunofluorescence single label

Table 1 presents the overall results for the immunofluorescent labeling of the various tissues used in these experiments. The cultured symbionts of the two species of *Aiptasia* showed positive labeling with the PC3 antibody. In those cultures that did show positive labeling, only a few cells were actually labeled among a larger population of unlabeled algal cells (Fig. 1a). However, none of the cultured symbionts showed any positive labeling with the G12 antibody (Fig. 1b). The symbionts extracted from *Cassiopeia* also did not show positive labeling to the PC3 antibody and appeared the same as negative controls (Fig. 1c, d). Extracted symbiosomes from *Aiptasia* showed very strong positive labeling to the PC3 antibody (Fig. 1e), whereas most of the labeling with the G12 antibody appeared to be in the contaminating host tissues (Fig. 1f).

Cryosectioned aposymbiotic tissues and pieces of tentacle from aposymbiotic animals all showed strong positive labeling for the G12 antibody (Fig. 2a). Neither sections nor whole mounts of aposymbiotic *A. pallida* showed any positive labeling with the PC3 antibody, and they appeared the same as negative controls (Fig. 2b). Sections and whole mounts of symbiotic *A. pallida* showed positive labeling with both the G12 antibody (Fig. 2c) and the PC3 antibody (Fig. 2d). As seen in Figure 2c, the G12 antigen was found

Table 1

Results of immunofluorescence imaging of cultured symbionts, extracted symbiosome, and whole tissue using PC3 and G12 antibodies

Tissue or cell tested	PC3 antibody	G12 antibody	Secondary antibody only	Nonspecific primary antibody
Cultured symbionts				
<i>Symbiodinium microadriaticum</i>	—	—	—	—
<i>S. pulchrum</i>	+	—	—	—
<i>S. bermudense</i>	+	—	—	—
Extracted symbiosomes from <i>Aiptasia pallida</i>	+	+	—	—
Cryosections				
Aposymbiotic tissue	—	+	—	—
Symbiotic tissue	+	+	—	—
Whole fixed tissue				
Aposymbiotic tissue	—	+	—	—
Symbiotic tissue	+	+	—	—

around the symbionts but was apparent in other portions of the host tissue as well. In contrast, as seen in Figure 2d, the PC3 antigen was found only directly around the symbionts and was not found throughout the host tissue.

Controls for cryosectioned symbiotic and aposymbiotic tissues and whole pieces of tissue that were incubated in either BM or a nonspecific primary antibody showed either no fluorescence in aposymbiotic tissues (Fig. 2b) or only the autofluorescence of symbiont chloroplasts (Fig. 2e).

#### Immunofluorescence double label

Double-labeled tissues (Fig. 2f) revealed the PC3 antibody labeling (green) to be confined to a very narrow band immediately surrounding the symbionts. Alternatively, G12 labeling

(red) was distributed evenly throughout the entire endodermal layer of the host and also appeared as a thin yellow line (the overlap color of red and green) that was immediately adjacent and external to the PC3 label (Fig. 2f). [Note that since the autofluorescence of the algal cell chloroplast is emitted at the same wavelength as the much brighter RITC label used for G12 identification, it was not possible to boost the gain and capture it. This resulted in the symbionts appearing as "black holes" (e.g., Fig. 2f), in contrast to what is seen in the single-labeling experiments (e.g., Fig. 2c).]

#### Immunogold single label

Table 2 summarizes the results of the immunogold labeling in these experiments. Standard fixed tissue and rapid-

**Figure 1.** Cultured symbionts and extracted symbiosomes from *Aiptasia pallida*, labeled with monoclonal primary antibodies PC3 or G12 and fluorescent secondary antibodies. Every picture, including the negative control, is a false color merger of green FITC labeling and red autofluorescence of the algal chloroplast. (a) Specific peripheral labeling of a minority of cultured *Symbiodinium bermudense* treated with PC3 antibody. (b) Total lack of labeling in cultured *S. bermudense* treated with G12 antibody. (c) Total lack of labeling in cultured *S. microadriaticum* treated with PC3 antibody. (d) Total lack of labeling in the negative control of cultured *S. bermudense* treated with secondary antibody only (all negative controls appeared the same). (e) Specific peripheral labeling of extracted symbiosomes from tissues of *A. pallida*, treated with PC3 antibody. (f) Extracted symbiosomes and contaminating host tissue from *A. pallida*, labeled with G12 and FITC. Scale bars = 20  $\mu$ m.

**Figure 2.** Cryosections and whole-tissue pieces of *Aiptasia pallida* labeled with PC3 antibody, G12 antibody, or both. Each picture, including the negative controls, is a merged image of green FITC labeling and red algal autofluorescence or RITC labeling. (a) General labeling of host tissue in a cryosection of aposymbiotic *A. pallida* treated with G12 antibody. (b) Total lack of labeling in a cryosection of aposymbiotic *A. pallida* treated with PC3 antibody. (c) General labeling of host tissue, as well as symbiosome membrane, in whole tissue from symbiotic *A. pallida* treated with G12 antibody. (d) Specific labeling of symbiosome-associated membranes without host tissue labeling in whole tissue from symbiotic *A. pallida* treated with PC3 antibody. (e) Total lack of labeling in a negative control of whole tissue from symbiotic *A. pallida* treated only with secondary FITC-conjugated antibody. (f) Double labeling of whole tissue from symbiotic *A. pallida* with PC3 (FITC—green) and G12 (RITC—red) antibodies; PC3 labeling is clearly limited to the symbiosome, whereas G12 labeling is mainly a characteristic of host tissue, but slightly overlaps the symbiosome, as represented by a thin yellow line surrounding the periphery of some symbiosomes. The black holes are the algal symbionts, the autofluorescence of which cannot be seen at the lower gain levels used to image the RITC signal. The bright orange spot in the center of many algal cells (c, d, e) is either the pyrenoid or the accumulation body of the cell. Data gathered using tissues labeled with acridine orange, a fluorescent nuclear stain, indicate that this structure is not the nucleus (Wakefield, unpubl. data). Scale bars: (a, b) = 50  $\mu$ m; (c–f) = 10  $\mu$ m.

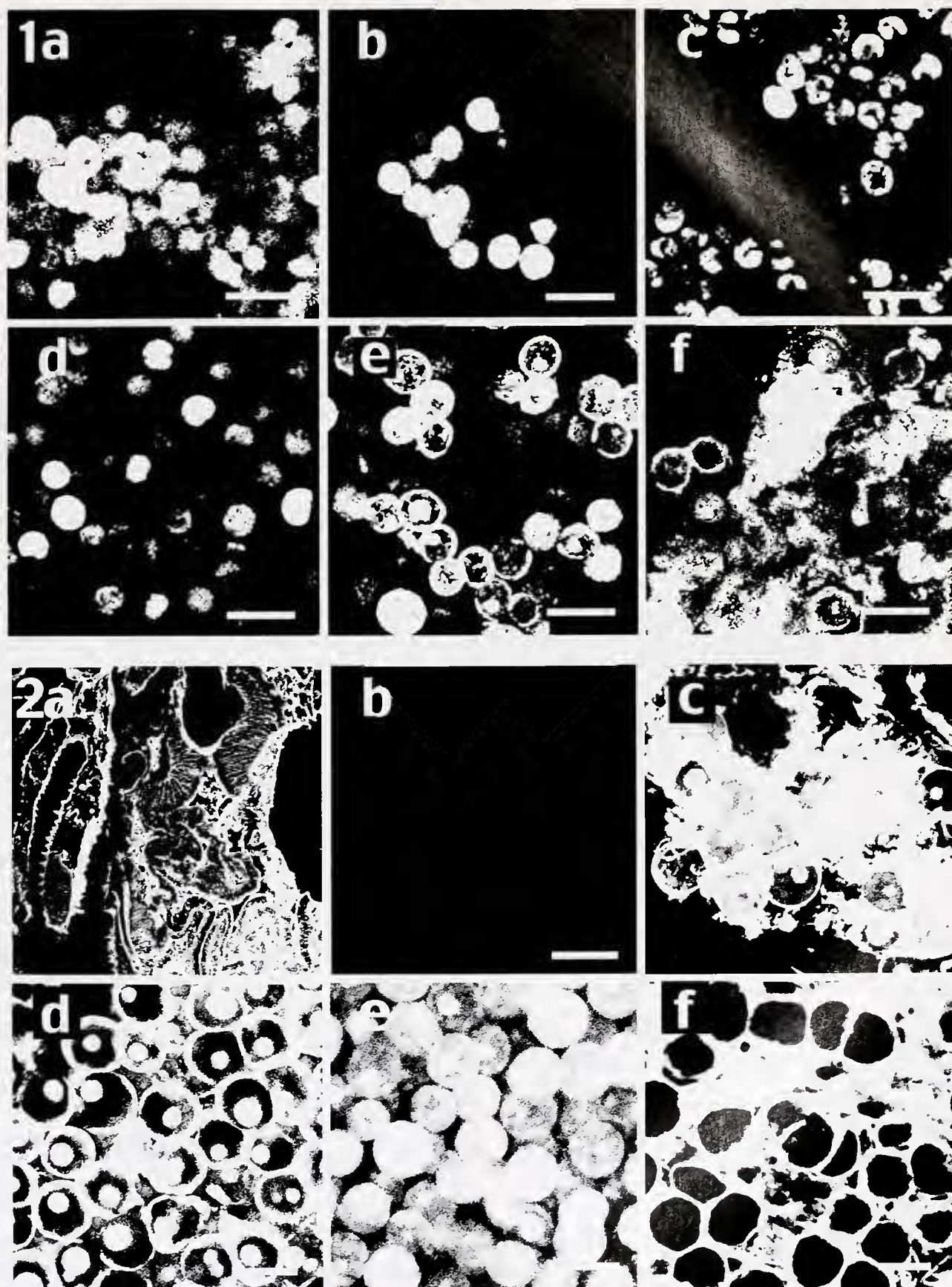


Table 2

*Results of immunogold labeling of in situ symbionts using PC3 and G12 antibodies*

Tissue components	PC3 label	G12 label	2° ab. only	Nonspecific primary	PC3 + IgG 2°	G12 + IgM 2°
Host tissues						
Ectoderm	—	—	—	—	—	—
Mesoglea	—	+	—	—	—	+
Endoderm	—	+	—	—	—	+
Symbiosome/Symbiont components						
Outermost symbiosome membrane	—	+	—	—	—	—
Multiple layers of membrane	+	—	—	—	+	—
Cell wall	—	—	—	—	—	—
Thecal vesicles	—	—	—	—	—	—
Cytoplasm	+	—	—	—	+	—

freeze-freeze-substituted-low-temperature-embedded tissue labeled with G12 and examined with the electron microscope showed positive labeling of the host cell plasma-membrane and internal membranes. In particular, the outermost symbiosome membrane was clearly labeled (Fig. 3a). There was also a strong labeling within the mesoglea of the host tissue (Fig. 3b). However, there was no labeling of the symbiont. A close examination revealed that the multiple layers of membrane that are immediately adjacent to the symbiont were not labeled by the G12 antibody (Fig. 3c); the ectodermal layer of the host tissue also lacked label. In fact, long extensions of the mesoglea could be traced into the ectodermal layer due to the strong label found therein and the absence of label within the ectodermal cells (Fig. 3b).

Controls that were incubated in either BM or a nonspecific primary antibody did not show positive labeling anywhere in the tissues (Fig. 3d).

The tissue sectioned by ultra-cryomicrotomy confirmed our findings that the G12 antigen indicated specific binding in the host membranes and mesoglea (Fig. 4a, b). It also revealed the specificity of the PC3 antibody to the algal symbiont (Fig. 4c, d). The labeling with the PC3 antibody was significant because our early attempts to label standard-fixed, LR-White-embedded tissue with PC3 had totally failed. Thus, the ultra-cryomicrotomy technique provided our first glimpse of the specificity of the PC3 antibody to the algal symbiont. Unfortunately, the preservation of ultra-structure in the ultra-cryomicrotomy technique was less than favorable, necessitating the use of the freeze-substituted-Lowicryl-embedding technique.

Labeling of the freeze-substituted-Lowicryl-embedded tissues with the PC3 antibody revealed strong labeling of the symbiont (Fig. 5a). Although there was a small amount of labeling present in the chloroplast and other cell organelles, the vast majority of label was found in the cytoplasm of the symbiont. Although the internal thecal vesicles and plates of the symbiont did not appear to be labeled, the PC3 antigen was concentrated around the periphery of the

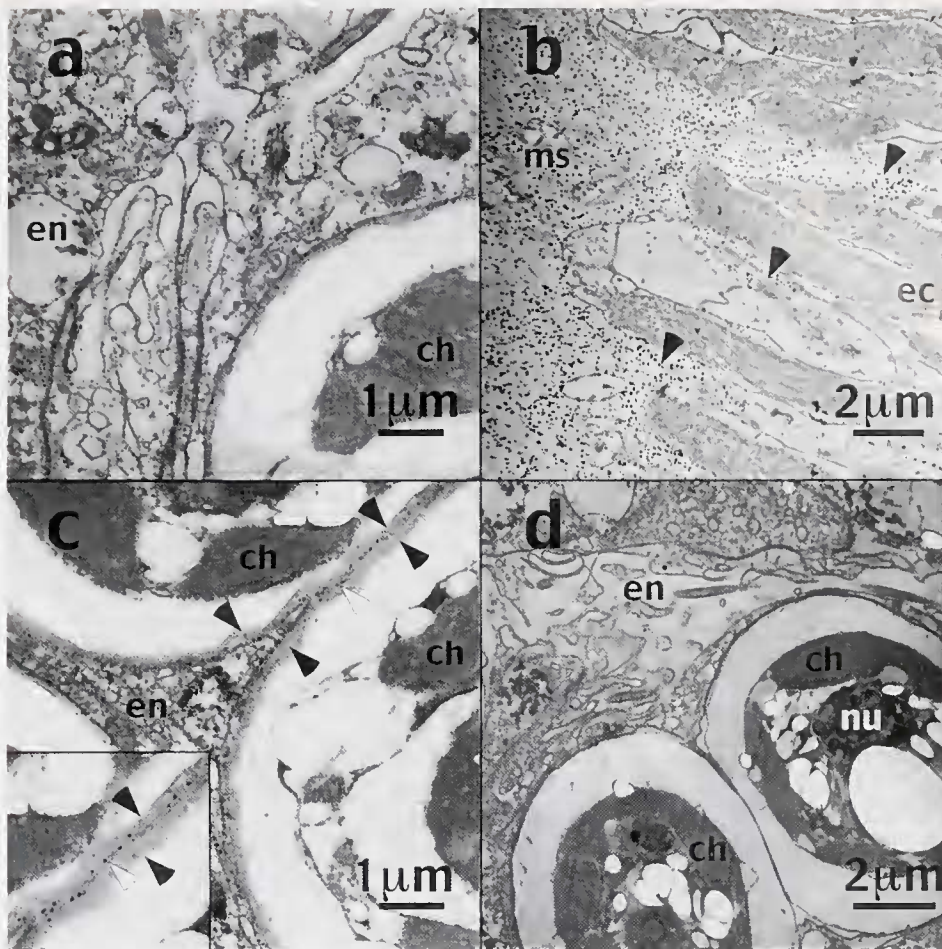
cell, between the chloroplast profiles (Fig. 5b). It was sometimes also seen in the cell wall directly adjacent to these peripheral cytoplasmic concentrations (Fig. 5b). Dense labeling was also present in the space between the symbiont and the outermost symbiosome membrane, including areas where multiple layers of membrane were present (Fig. 5b). Host cell cytoplasm and associated membranes surrounding the symbiosomes were essentially devoid of label (Fig. 5a). The minute amount of labeling in the host cell cytoplasm was similar to that of the negative controls (Fig. 5c) and was thus considered to be background labeling.

#### *Immunogold double label*

Tissues labeled with both primary antibodies and different-sized gold-conjugated secondary antibodies confirmed our single-labeled experiments. The outermost symbiosome membrane, host endodermal cell membranes, and mesoglea showed dense labeling with large gold particles, indicating the presence of the G12 antigen. Conversely, the multiple layers of membrane directly adjacent to the cell wall, the cytoplasm between the chloroplast profiles, and the cell wall adjacent to these cytoplasmic areas were heavily labeled with small gold particles, indicating the presence of the PC3 antigen (Fig. 6a, b). Negative controls in which the primary and secondary antibodies were switched did show a small amount of cross reactivity. This was most evident with PC3 and the IgG-specific antibody, perhaps due to its larger particle size. However, the cross reaction of the IgG-specific antibody to PC3 confirmed that the PC3 antibody was specific for the algal cell (Fig. 6c). There was some cross reactivity of the IgM antibody to G12, but this also confirmed that the G12 antibody was specific for host tissue and not for the algal cell.

#### **Discussion**

The results of both the immunofluorescent and immunogold labeling experiments clearly support the hypothesis

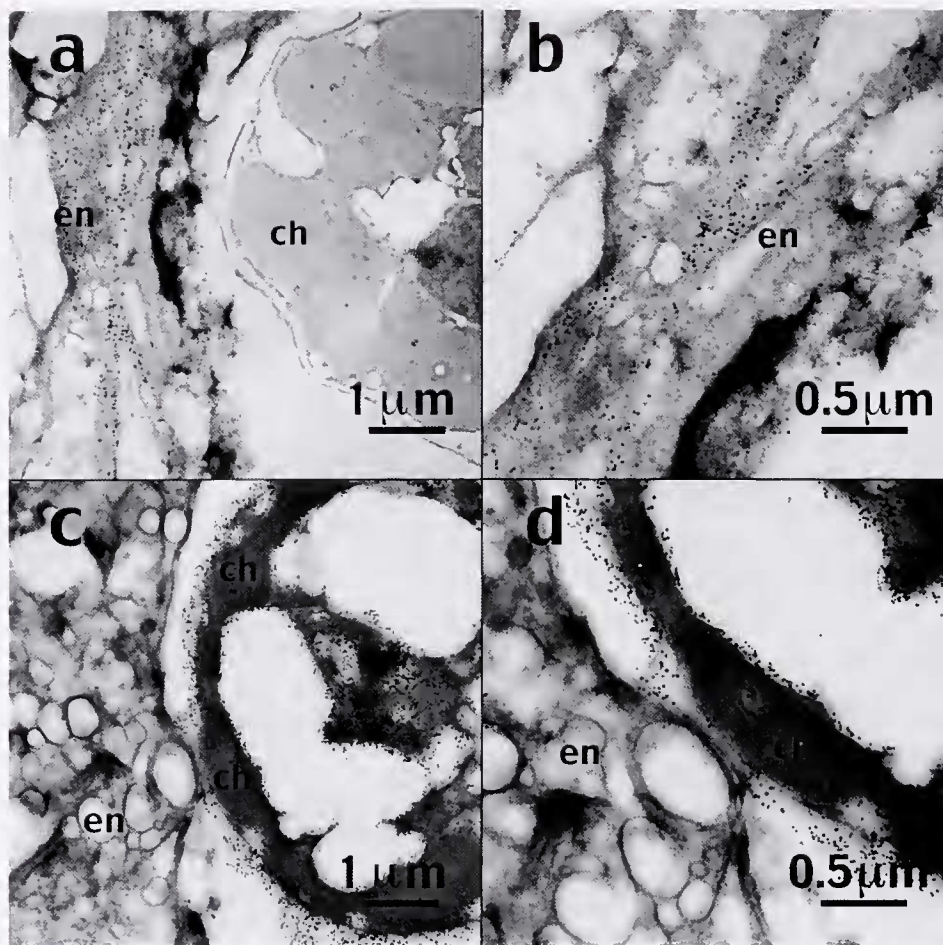


**Figure 3.** Labeling of host membranes in plastic-embedded tissues of symbiotic *Aiptasia pallida* treated with G12 antibody and visualized with immunogold particles. (a) General labeling of host cell membranes in standard aldehyde-fixed and dehydrated *A. pallida*, embedded in LR White (G12 visualized with protein A–15-nm gold particles). (b) Extension of labeled mesoglea (arrowheads) into unlabeled ectoderm in freeze-substituted *A. pallida*, embedded in Lowicryl (G12 localization visualized with silver-enhanced ultrasmall gold particles). (c) Labeling of the outermost symbiosome membrane in standard aldehyde-fixed and dehydrated *A. pallida*, embedded in LR White (G12 visualized with protein A–15-nm gold particles); insert shows labeling of the outermost membranes of two adjacent symbiosomes. Note that host membrane (white arrowhead) is labeled, but inner, symbiont-associated membranes (black arrowheads) are not. (d) Lack of specific labeling in a negative control section of standard aldehyde-fixed and dehydrated *A. pallida* tentacle embedded in LR White, incubated without G12 antibody followed with incubation with protein A–15-nm gold particles; all negative controls appeared the same with only minor background scattering of gold particles. ch = chloroplast, ec = ectodermal cells, en = endodermal cell cytoplasm, ms = mesoglea, nu = nucleus.

that the two monoclonal antibodies, G12 and PC3, are specific for the different partners in this symbiotic association. The fact that G12 shows positive labeling of host tissue in whole mounts and sections of *Aiptasia pallida* and no labeling of cultured symbionts demonstrates that it is binding to some antigen that is found exclusively in host tissue. Also, since it shows positive labeling of both symbiotic and aposymbiotic tissues, it is clear that this antigen is not symbiont-induced and is present in the host tissue regardless of its symbiotic condition. By contrast, PC3 labeling is apparent only in symbiotic tissues and is completely absent

in the aposymbiotic condition. One possible explanation is that the PC3 antigen is induced in the host by the presence of the symbionts and then somehow transferred to them. However, since *in vitro* cultured *Symbiodinium bermudense* and *S. pulcherrimum* also show positive (albeit reduced) labeling with the PC3 antibody, it is clear that this antigen is symbiont-derived.

The finding that cultured *S. microadriaticum* is not labeled by the PC3 antibody but both *S. pulcherrimum* and *S. bermudense* are labeled may reflect phylogenetic differences between the three tested algal species. In past studies,

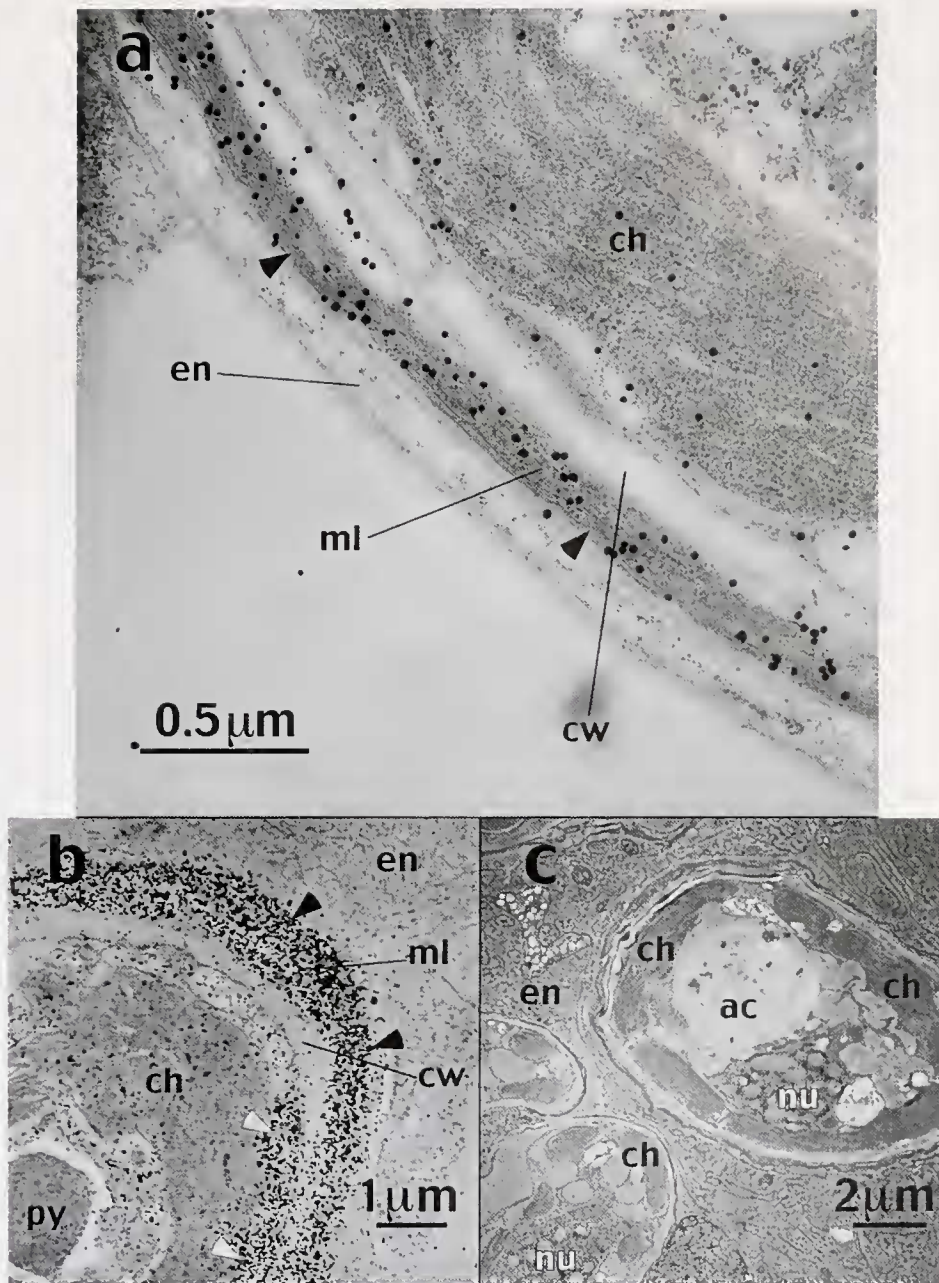


**Figure 4.** Ultra-cryomicrotomy-sectioned symbiotic *Aiptasia pallida* labeled with G12 or PC3 and immunogold particles indicating the specificity of the antibodies to their respective tissues. Fixation quality varied greatly, but cell morphology was adequate to distinguish host *versus* symbiont. (a) Host endodermal cell cytoplasm labeled in tentacle tissue of *A. pallida*, incubated with G12 and an IgG-specific secondary antibody conjugated to ultrasmall gold particles. Note that algal symbionts are not labeled. (b) Higher magnification of the ultra-cryomicrotomy-sectioned *A. pallida*, labeled with G12 and an IgG-specific secondary antibody conjugated to an ultrasmall gold particle (1:10). (c) Labeling of algal symbionts and associated material in tentacle tissue of *A. pallida* incubated with PC3 and an IgM-specific secondary antibody conjugated to an ultrasmall gold particle. Note the labeling of material between the symbiont and the host cytoplasm as well as internally within the algal symbiont. Label within the symbiont is localized in the cytoplasm surrounding the chloroplasts. Host cytoplasm is devoid of label. (d) Higher magnification of the ultra-cryomicrotomy-sectioned *A. pallida*, labeled with PC3 and an IgM-specific secondary antibody conjugated to an ultrasmall gold particle (1:10). ch = chloroplast, en = endoderm.

evidence based on karyotyping (Blank and Trench, 1985), morphology (Schoenberg and Trench, 1980b; Trench and Blank, 1987), isoenzyme patterns (Schoenberg and Trench, 1980a), physiology (Fitt and Trench, 1981; Chang *et al.*, 1983; Fitt and Trench, 1983), and biochemistry (Chang and Trench, 1982; Govind *et al.*, 1990) has indicated that the genus *Symbiodinium* consists of many different species. Also, an examination of similarities and differences in the genes that encode for the small ribosomal subunit of algal symbionts has revealed that *S. bermudense* and *S. pulchrum* are closely related but different species, and both have been placed into clade B of the "zooxanthellae" phyloge-

netic tree (Rowan, 1998). *S. microadriaticum*, on the other hand, is more distantly related and has been identified as belonging to clade A (McNally *et al.*, 1994; Rowan, 1991, 1998; Rowan and Powers, 1991). The results of our immunofluorescent studies support the concept of phylogenetic diversity among the symbionts and may be added to the list of biochemical differences that exist among some *Symbiodinium* species.

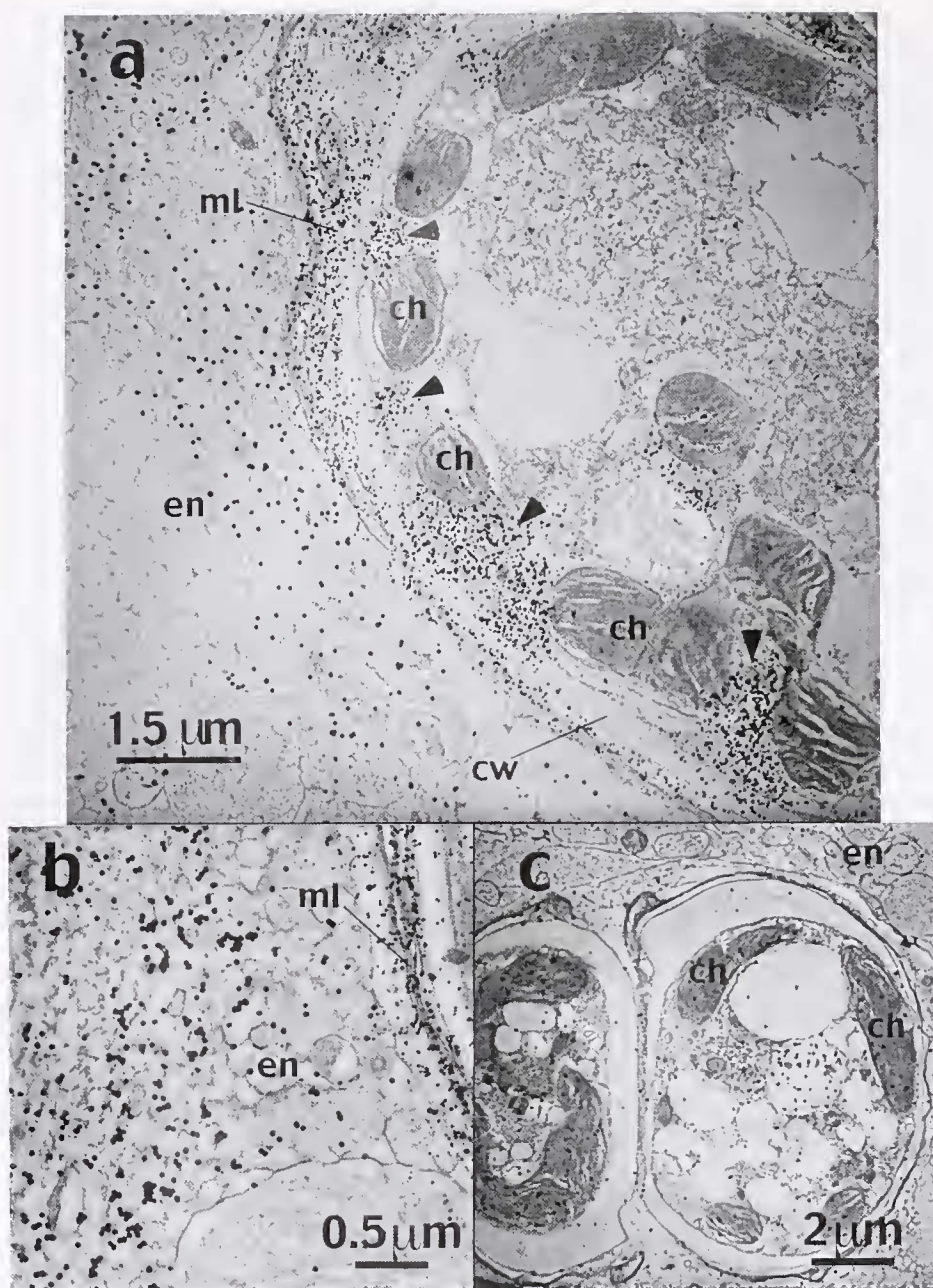
The double-labeled immunofluorescent and immunogold results clearly demonstrate that the algal symbionts are surrounded by layers of material that originate from the different symbiotic partners. The narrow region of PC3



**Figure 5.** Freeze-substituted symbiotic *Aiptasia pallida* labeled with PC3 and an IgM-specific secondary antibody conjugated to an ultrasmall gold particle. The fixation of this tissue was extremely good for EM cytochemical studies. Both host and symbiont tissues were well preserved, and distinct morphological features were easily discernible. (a) Freeze-substituted symbiotic *A. pallida* tissue labeled with PC3 and ultrasmall gold antibody (1:100); arrowheads indicate label of multiple layers of membrane; note the absence of label in either the host endodermal layer or the symbiont's cell wall. (b) Specific labeling of symbiont membranes and no host-tissue labeling in freeze-substituted *A. pallida* labeled with PC3 and ultrasmall gold antibody (1:10); arrowheads indicate heavily labeled multiple layers of symbiont membranes; white arrowheads indicate abundance of PC3 antigen at the periphery of the algal cell. (c) Freeze-substituted *A. pallida* tissue negative control; blocking media followed by ultrasmall gold antibody (1:10); all negative controls appeared the same, with only minor background scattering of gold particles. ac = accumulation body, ch = chloroplast, cw = cell wall, en = host endodermal tissue, ml = multiple layer of symbiont membranes, nu = nucleus, py = pyrenoid.

labeling that is peripheral to the symbionts and is surrounded by a single, host-derived "symbiosome membrane" and also by a large ubiquitous area of G12 labeling illus-

trates that a limited layer of this symbiont-derived antigen is maintained within a vast amount of host tissue. The immunogold results indicate that the PC3 antigen is present in the



**Figure 6.** Freeze-substituted symbiotic *Aiptasia pallida* double labeled with both G12 and PC3 and with IgG-specific-antibody-20-nm gold particle and IgM-specific-antibody-ultrasmall gold particle. (a) Note the difference in particle size between the host membranes and the symbiont membranes and cytoplasm that clearly defines the distribution of the G12 (large particles) and PC3 (small particles) antigens; arrowheads indicate abundance of PC3 antigen at the periphery of the algal cell, particularly between the chloroplast profiles. (b) Higher magnification of the freeze-substituted symbiotic *A. pallida* double label, clearly showing the PC3-labeled symbiont membranes and the G12-labeled host membranes. (c) Freeze-substituted symbiotic *A. pallida* cross reactivity control in which PC3 was incubated with the IgG-specific secondary antibody; the slight cross reactivity of this nonspecific secondary antibody supports the assertion that PC3 is an algal-cell-specific antibody (note the lack of label in the host tissues). ch = chloroplast, cw = cell wall, en = host endodermal tissue, ml = multiple layer of symbiont membranes.

algal cell wall, but only in areas directly adjacent to high cytoplasmic levels of this antigen. This suggests that these may be areas of secretion for a molecule or molecules

containing the PC3 antigen. The fact that the PC3 antigen is found both within the algal cell cytoplasm and also closely associated with the multiple layers of membrane, beneath

the G12-labeled host symbiosome membrane, supports the hypothesis that these membranous layers are of symbiont rather than host origin.

The results of this research should help clarify some confusing terminology currently used in the field of cnidarian–dinoflagellate endosymbiosis. The term “symbiosome,” as introduced by Roth *et al.* (1988), was defined as “a membrane-bound compartment containing one or more symbionts and certain metabolic components located in the cytoplasm of eukaryotic cells.” They further describe the “symbiosome membrane” as being a host-derived membrane that surrounds the microsymbiont, creating a “symbiosome space” (Roth *et al.*, 1988). This terminology has been adopted by researchers in various fields of endosymbiotic research, including plant–bacteria interactions (Day and Udvardi, 1992; Udvardi and Day, 1997), protozoan–bacteria interactions (Choi and Jeon, 1992; Jeon, 1992), and invertebrate–algal interactions (Rands *et al.*, 1993; Wakefield *et al.*, 1998; Wakefield *et al.*, 2000). For plant–bacterial and protozoan–bacterial interactions, these terms are adequate and clearly define the endosymbiotic situation of these partners. However, for invertebrate–algal symbioses, particularly cnidarian–dinoflagellate symbioses, use of the term “symbiosome membrane” has been problematic.

*In situ* dinoflagellate symbionts have been identified as existing predominantly in a coccoid, or vegetative, state within the host cells (Trench and Blank, 1987). The coccoid state is defined by the presence of the spherical “pellicle,” or cell wall. However, exterior to the cell wall exist multiple layers of apparent membranes. Historically, these layers have been referred to by a variety of terms, including the periplast (Dodge and Crawford, 1970; Kevin *et al.*, 1969; Palincsar *et al.*, 1988; Taylor, 1968; Trench and Blank, 1987), the amphisma (Schoenberg and Trench, 1980a; Thinh *et al.*, 1986), the theca (Battey, 1992; Taylor, 1971), the vacuolar membranes (Colley and Trench, 1983), or simply the cell covering (Tripodi and Santisi, 1982). The origin of these membranes has also been debated for quite some time.

Tripodi and Santisi (1982), working with the symbiotic octocoral *Eunicella stricta*, assigned the multiple membranes to the host cell. They stated that the location of recognizable host organelles on the “inner side” of the membranes indicated that, in this host species, the membranes were composed of host plasmalemma. They also stated that the “many times folded” plasmalemma would have an increased surface area and thus could have a functional significance; however, they did not relate what this might be, nor did they indicate how the plasmalemma would accomplish this folding process (Tripodi and Santisi, 1982). Palincsar *et al.* (1988), working with *Aiptasia pallida*, also assigned the multiple membranes to the host but suggested that they were formed from internal host cell membranes rather than from the plasmalemma. They hypothesized that

membrane-bound cytoplasmic extensions of host tissue could envelope the symbiont, followed by the extrusion of the cytoplasm. This would produce the multiple layers of membrane with no cytoplasmic separations (Palincsar *et al.*, 1988). Other authors, including Colley and Trench (1983) and Rands *et al.* (1993), have also assigned the origin of the multiple membranes to the host cell.

On the other hand, Kevin *et al.* (1969), Schoenberg and Trench (1980b), Trench and Blank (1987), and Muller-Parker *et al.* (1996) have identified a single host-derived membrane and assigned the origin of the multiple layers of membrane to the algal symbiont. Most do so without postulating a mechanism for how these membranes might be produced; however, Trench and Blank (1987) have suggested one possibility. They stated that the cell wall of symbionts is covered by an acidic polysaccharide or glycoprotein layer that resembles a membrane at the ultrastructural level, but in fact contains no lipids. They further suggested that this layer is occasionally sloughed off the outer surface of the cell wall. As these layers accumulate, they appear as multiple membranes exterior to the cell wall and produce the peripheral scroll-like layers of apparent membrane often seen in ultrastructural sections (Trench and Blank, 1987).

In earlier papers (Wakefield *et al.*, 1998, 2000), we also suggested that the membranes were derived from algal cells, but we proposed a very different mechanism for the production of these membranes than that put forth by Trench and Blank (1987). Briefly, we hypothesized that the algal symbionts were continuing through their normal ecdysis cycle within the host cell vacuole. By cycling through the coccoid-to-thecate stage and back again, a layer of apparent multiple membranes would accumulate between the host cell symbiosome membrane and the symbiont. The membranous layers would be composed of the sloughed plasma membrane, thecal vesicles, and plates from each ecdysis event. Therefore, we feel that any assignment of the multiple layers of membrane to the host (with the exception of the single outermost symbiosome membrane) is unsubstantiated and probably in error.

If the host-derived origin for the membranes were correct, then each and every membrane in the layer could be defined as the “symbiosome membrane,” as defined by Roth *et al.* (1988). However, the results of labeling with our PC3 antibody indicate that the multiple layers of membrane lying between the host vacuole membrane and the symbiont cell wall originate from the algal symbiont and are held in place by the presence of a single host-derived membrane. Therefore, the host-derived membrane is the definitive symbiosome membrane, while the other apparent membranes could be defined as symbiont-derived “metabolic components” within the symbiosome space (see Roth’s [1988] original definition of the symbiosome). However, designating these membranes as mere “metabolic components” may belie

their potential importance to the symbiotic system. In subsequent text, the term "symbiont membranes" is used to describe these structures.

On the basis of the presence of the PC3 antigen within the symbiont membranes and the cytoplasm, it may be hypothesized that, during the ecdysis process, cytoplasmic material containing the PC3 antigen is also shed and becomes trapped between the layers of plasmalemma and thecal vesicles described by Wakefield *et al.* (2000). Another possibility is that the PC3 antigen is simply a substance exuded through the cell wall during the coccoid stage of the life cycle. This material could accumulate and become trapped between the layers of shed plasmalemma and thecal vesicles during ecdysis. Alternatively, if Trench and Blank (1987) are correct, exuded PC3 antigen would become trapped between the layers of polysaccharide or glycoprotein material that is occasionally sloughed from the outer cell wall.

Although the identification of the PC3 and G12 antigens is still under investigation in our laboratory, recent data indicate that the PC3 antigen is, in fact, an exuded material (Wakefield and Kempf, unpubl. data). Markell and Trench (1993) have demonstrated that cultured symbionts do exude large water-soluble glycoconjugates. If this exudate is produced *in situ* (in the symbiotic state), then these molecules could provide essential nutrients to the host (provided the host could digest them), and might also serve as molecular signals between the host and the symbiont (Markell and Trench, 1993).

The potential of the symbiont membranes (or trapped exudates) to act as molecular signals is suggested by the findings of Colley and Trench (1983). They performed a series of experiments in which freshly isolated symbiosomes from *Cassiopeia xamachana*, *Aiptasia pallida*, *A. pulchella*, *Anthopleura elegantissima*, and *Zoanthus* sp. were introduced into the gastrovascular cavity of aposymbiotic scyphistomae of *C. xamachana*. They also introduced cultured algae isolated from the cnidarian hosts *C. xamachana*, *A. pallida*, *A. tagetes*, and *Zoanthus sociatus*. They found that all cultured algae, even those that were specific for *C. xamachana*, were either completely rejected or phagocytosed at almost immeasurably low rates. By contrast, the freshly isolated symbiosomes from each host species were all phagocytosed at relatively rapid rates. When viewed with the transmission electron microscope, all of the freshly isolated symbionts were surrounded by multiple layers of apparent membrane. These membranes were completely absent from the cultured algal cells. After several experiments, the authors concluded that a recognition process must be occurring at the surface between the host endodermal cell and the specific membrane layers that surround the algal symbiont (Colley and Trench, 1983).

Believing that there were no algal cell membranes exterior to the cell wall, Colley and Trench (1983) assigned the

origin of these membranes to the host and called them "vacuolar membranes." They hypothesized that the enhanced uptake of freshly isolated symbiosomes was a result of the presence of these host membranes, and that there must be a specific molecular factor involved. Since the host endodermal cell was making its initial contact with various "host membranes" of injected symbiosomes, they concluded that there was no actual intercellular (host-symbiont) recognition occurring, and that the uptake of symbiosomes might be indistinguishable from the phagocytosis of food particles.

As we have clearly demonstrated in this study, in *Aiptasia pallida* there are a multitude of symbiont-produced membranes or membrane-like layers that surround the algal cell wall and are associated with high concentrations of the symbiont-derived PC3 antigen. We postulate that the rapid uptake of isolated symbiosomes compared to the low uptake of cultured algae observed by Colley and Trench (1983) may have resulted from the absence of the correct molecular signal at the external surface of most cultured algal cells. Since our results reveal the PC3 antigen to be absent from most (but not all) suspended, cultured algal symbionts from *A. pallida* and *A. pulchella* and present in all symbiosomes *in situ*, it is possible that such an antigen may be the actual signal that stimulated rapid phagocytosis in the experiments of Colley and Trench (1983). Similarly, since cultured symbionts lack the multiple layers of symbiont membranes found *in situ*, it is also possible that these membranes may contain the molecular signal that stimulates phagocytosis. We feel that it would be useful to reexamine the question of host recognition of algal symbionts with attention to these possibilities.

It is unknown how aposymbiotic cnidarian offspring that must recruit their symbionts from the environment achieve an initial infection (Berner *et al.*, 1993; Davy *et al.*, 1997; Schwarz *et al.*, 1999); however, there is some support for the hypothesis that the motile thecate stage of the symbiont rather than the sessile coccoid stage is capable of infecting (Kinzie, 1974). If the plasmalemma of the thecate stage contains the appropriate molecular signal, these algal cells would be readily phagocytosed, thus resulting in the initial infection. If such signal molecules can stimulate phagocytosis, then other host- or symbiont-specific molecules must be involved in maintaining the symbiosis once it is established (Trench, 1993). Although Colley and Trench (1983) found that all of the freshly isolated symbionts were phagocytosed by *Cassiopeia xamachana* scyphistomae, only *Symbiodinium microadriaticum* and *S. bermudense* were ultimately maintained in the endodermal cells and could stimulate strobilation. Therefore, the signals that stimulate phagocytosis could not be the same as those that maintain the symbiosis.

Host-symbiont-specific signaling has been shown to occur in many other symbiotic associations such as the *Rhi-*

zobium-legume association (Lerouge *et al.*, 1990; Sanchez *et al.*, 1991), pathogenic-bacteria-plant interactions (Dixon and Lamb, 1990; Regensburg-Tuink and Hooykaas, 1993), *Mycobacterium*-macrophage invasions (Ferrari *et al.*, 1999), X-bacteria-amoeba symbiosis (Jeon, 1992, 1997), parasite-host infections (Sibley *et al.*, 1986; Sibley and Krahenbuhl, 1988; Perkins, 1989; Sam-Yellowe, 1992; Collins *et al.*, 1997), and diatom-foraminifera interactions (Lee *et al.*, 1988, 1997; Lee, 1998; Chai and Lee, 1999, 2000). In many of these associations, the specific signals leading to successful symbiotic interactions have been elucidated. *Agrobacterium tumefaciens*, a bacterium that induces tumors in many types of plants, is dependent upon the VirF protein for tumor formation (Regensburg-Tuink and Hooykaas, 1993). *Plasmodium falciparum* uses several proteins, including the 155-kDa ring-infected surface antigen and the 140/130/110-kDa rhoptry complex, to bind to appropriate erythrocyte membranes (Sam-Yellowe, 1992). *Rhizobium meliloti* uses a  $\beta$ -1,4-tetrasaccharide of D-glucosamine to induce root nodules in alfalfa (Lerouge *et al.*, 1990). *Mycobacterium* species use the TACO protein to inhibit lysosomal fusion in macrophages (Ferrari *et al.*, 1999). The frustules of all endosymbiotic diatoms of large foraminifera display a 104-kDa polypeptide that prevents their digestion by the host (Chai and Lee, 1999).

Attempts to locate and characterize similar types of molecular signals required for establishing and maintaining zooxanthella-cnidarian symbioses have met with little success; however, the two reported attempts to identify these signals concentrated on the algal cell wall (Markell *et al.*, 1992) and on water-soluble glycoconjugates released by algal cells in culture (Markell and Trench, 1993). Because we have identified the symbiont membranes to be of algal-cell origin and intimately associated with a symbiont-derived antigen (PC3), and because an earlier investigation (Kinzie, 1974) supports the hypothesis that molecular signals associated with the thecate motile stage must stimulate phagocytosis, it may be hypothesized that these symbiont membranes are involved in this signal transduction event. It is likely that knowledge of the identity and function of such molecular signals will be critical to our understanding of the cellular mechanisms orchestrating events such as coral bleaching (Bunkley-Williams and Williams, 1990; Ghiold, 1990; Gleason and Wellington, 1993; Goreau and Hayes, 1994; Brown *et al.*, 1995), where such signaling capabilities have apparently been lost.

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